AWARD NUMBER: W81XWH-14-1-0500

TITLE: **Towards a Molecular Understanding of Noise-Induced Hearing Loss**

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REPORT DATE: October 2015

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual Report	30Sep2014-29Sep2015

4. TITLE AND SUBTITLE

Towards a Molecular Understanding of Noise-Induced Hearing Loss

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0500

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland
Baltimore, MD 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project is to understand the inner ear cell type-specific changes induced by different types of noise and pre-conditioning interventions. To date, we have calibrated the noise exposures, and successfully completed tissue collection and processing for RNA-seq of baseline and noise treated samples at two different time points following noise exposure. We also established the sequencing conditions for all libraries and have been critically comparing the pre-conditioning treatments to carefully select the ones for further investigation.

15. SUBJECT TERMS
Permanent threshold shift, Temporary threshold shift, Noise induced hearing loss, Ribotag, RNA-seq, hair cell, supporting cell

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	21	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Noise induced hearing loss (NIHL) is a major health concern for the Department of Defense. Noise exposure often is inevitable, and may results in a permanent loss of hearing. Unfortunately, there are no treatments to prevent or reverse NIHL. As a first step towards designing targeted therapeutics, we suggested to generate mouse models which allow for cell type-specific translatome analysis in the ear. These, in turn, will be used to analyze the genes expressed in the hair cells (HC) and supporting cells (SC) of adult mice before and after different types of noise exposure as well as pre-conditioning treatments, which in mice, can ameliorate NIHL. Here we report our progress over the first year of the project, which was focused on generating the mouse models, calibrating the systems for noise exposure and translatome analysis and tissue collection for baseline and exposure of noise which leads to a permanent threshold shift.

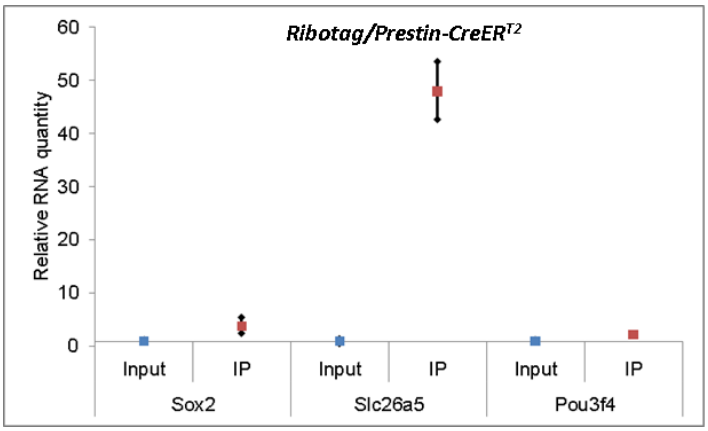
KEYWORDS

Permanent threshold shift, Temporary threshold shift, Noise induced hearing loss, Ribotag, RNA-seq, Hair cell, Supporting cell

ACCOMPLISHMENTS

Major goals of the project and their accomplishments:

I. Specific Aim 1: To determine the OHC- and SC-specific transcriptional and signaling cascades activated in vivo in response to PTS-inducing noise exposure
   a. Major Task 1: To establish the OHC- and SC-specific translatome of adult mouse inner ears. Progress by subtasks:
      i. Obtain ACURO approval following UMSOM IACUC approval – this was obtained, however, in a three-month delay due to a delay in processing by the DOD. Therefore, the task was complete on month 4 rather than month 2 and animal work began thereafter.
      ii. Mouse crosses and tissue harvesting – complete.
      iii. Tissue processing – complete 100%, polysome IP – complete 100%, submission of samples for RiboTag-seq – complete 100%; RiboTag-seq – libraries have been constructed, sequencing pending (50% complete). Appropriate transcript enrichment obtained after polysome IP is tested by a real time RT-PCR assay calibrated to routinely assess project samples (Fig. 1).



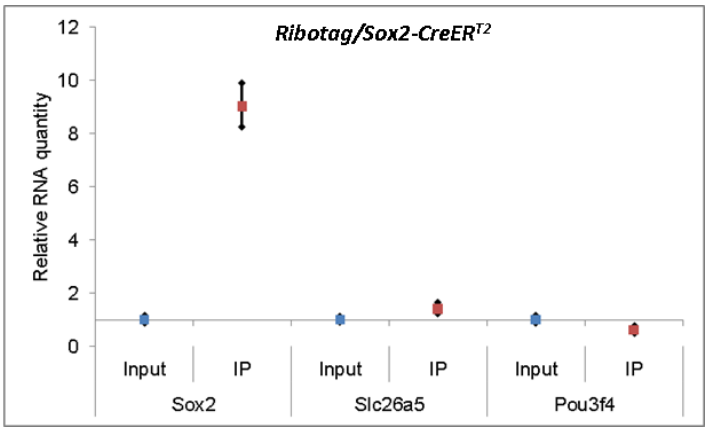
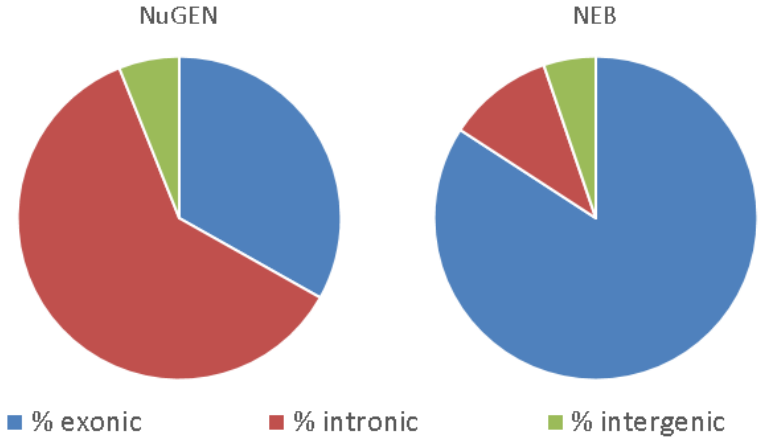


Figure 1 – gene-specific enrichment in the immunoprecipitated (IP) samples from the *Prestin-CreER^{T2}* (left) and *Sox2-CreER^{T2}* (right) mice. Graphs represent the relative quantity (RQ) of transcripts in the IP compared with the input. Three genes are assayed – *Sox2* specific for supporting cells in adult mice, *Slc26a5* (encodes for Prestin) to test for OHC translatome enrichment, and *Pou3f4* for non-epithelial cells. A robust enrichment for *Slc26a5* and *Sox2* is obtained in the IP RNA from the *Ribotag/Prestin-CreER^{T2}* and *Ribotag/Sox2-CreER^{T2}*, respectively.

Of note, calibration experiments showed that our standard RNA-seq protocol using the NuGEN Ovation kit resulted in over-representation of introns. A second calibration experiment using a variety of kits was added and the NEB kit was found to have superior results (Fig. 2). This experiment is now being repeated using four different kits and we plan to publish its results as a guide to the community on tissue processing from RiboTag mice.

Figure 2 – Dramatic over-representation of intronic sequences in RiboTag-IP libraries prepared for sequencing with the NuGEN amplification kit is resolved when using the NEB kit to prepare the libraries.



iv. Data analysis – 0% (was scheduled for months 6-9, however, due to delay in starting animal work and pace of mouse crosses, project is 3-6 months delayed).

v. Validation experiments – scheduled originally for months 8-12 and 31-36. Still pending.

b. Major Task 2: To determine the OHC- and SC-specific transcriptional and signaling cascades activated in response to PTS-inducing noise injury. Progress by subtasks:

i. Mouse crosses, noise exposure, tissue harvesting, histological analysis, ABR and DPOAE measurements. Scheduled for months 3-6, performed in months 4-12. Task is 100% compete for the *Ribotag/Prestin-CreER^{T2}* and 70% complete for *Ribotag/Sox2-CreER^{T2}*.

1. Noise exposures were calibrated to generate a PTS in the *Ribotag/Prestin-CreER^{T2}* and *Ribotag/Sox2-CreER^{T2}* (Fig. 3).

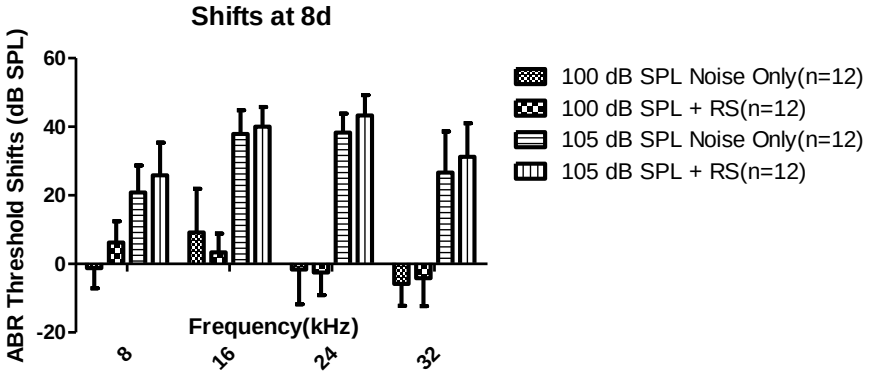


Figure 3 – ABR threshold shifts 8 days after 100 and 105 dB SPL noise exposure, with or without restraint stress (RS). 105 dB SPL produces a stable threshold shift throughout the length of the cochlear duct and was therefore chosen for characterization of PTS. (n=number of ears).

2. ABRs performed on the control mice and cytocochleograms were completed on four representative ears. Outer hair cell (OHC) loss was measured by staining whole mounted tissues with antibody for Prestin. All cell nuclei were labeled with DAPI, and the actin cytoskeleton was visualized using phalloidin (Fig. 4).

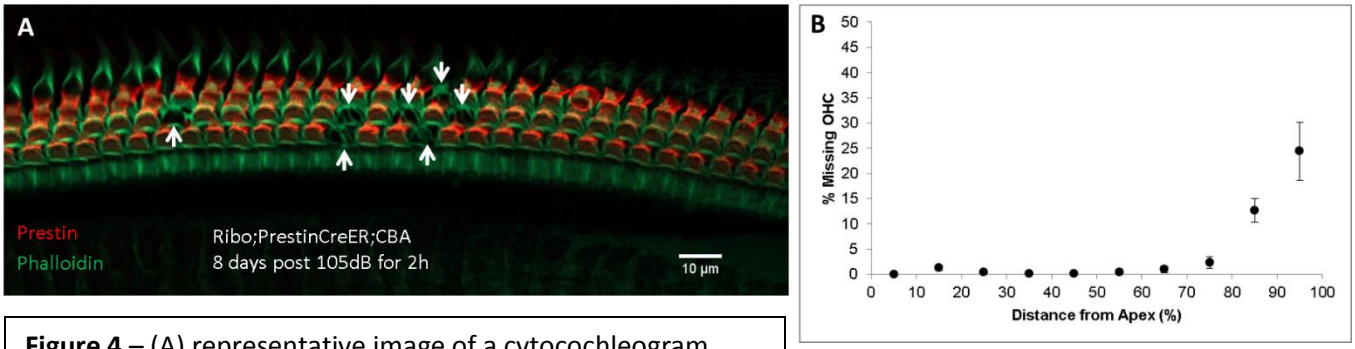


Figure 4 – (A) representative image of a cytocochleogram. The area shown is between 70% and 80% from the apex; (B) Average hair cell loss as tested from four mice after 8-16 kHz 105 dB SPL, 2 hour noise exposure.

ii. Tissue processing – 100% complete for *Ribotag/Prestin-CreER^{T2}* and 70% complete for *Ribotag/Sox2-CreER^{T2}*, polysome IP - 100% complete for *Ribotag/Prestin-CreER^{T2}* and 70% complete for *Ribotag/Sox2-CreER^{T2}*and RiboTag-seq - – libraries have been constructed, sequencing pending (100% complete for the *Ribotag/Prestin-CreER^{T2}*), this task is still pending completion of tissue harvesting for the *Ribotag/Sox2-CreER^{T2}*.

iii. Data Analysis and Validation – pending sequencing.

II. Specific Aim 2: To determine the OHC- and SC-specific signaling cascades activated in vivo in response to otoprotective interventions.

a. Subtask 1: This task was greatly expanded to first test results of the various noise exposures as well as pre-conditioning treatments in our strain, as suggested by the study section. In the original project proposal we planned to use the *Ribotag/Prestin-CreER^{T2}* and *Ribotag/Sox2-CreER^{T2}* in their original background, which is C57BL/6. Per study section request, the *Ribotag/Prestin-CreER^{T2}* and *Ribotag/Sox2-CreER^{T2}* were crossed to CBA mice to avoid a homogeneous C57BL/6 background. Therefore, all mice would now be 50% C57BL/6 and 50% CBA/CAJ. For calibration experiments, and to avoid loss of experimental animals, B6CBAF1/J mice were purchased from the Jackson Laboratories, shipped to the University of Maryland School of Medicine age 7-8 weeks and tested at 10 weeks of age.

i. Determining the conditions for a PTS in the B6CBAF1/J and testing efficacy of restraint stress as a pre-conditioning treatment. Our data indicate that octave-band noise exposure (8-16 kHz for 2 hours) at 100 dB SPL induced a TTS in the B6CBAF1/J mice while 105 dB SPL induced a PTS in the B6CBAF1/J mice. Pre-treating the mice with 2 hours of restraint stress did not change the outcome of the noise exposure. All noise exposures were performed at the same time of day (8am) and all groups consisted of 50% male and 50% female mice (Fig. 3). We concluded that 105 dB SPL induces a definite PTS, and therefore used this condition for Major Task 2 in Specific Aim 1. We also concluded that the effect of a 2-hour restraint stress is marginal and proceeded to test the effect of 2, 4 and 6-hour restraint stress as a pre-conditioning treatment for a 105 dB SPL noise exposure. At the completion of these experiments we found that secondary to a problem in the animal facility, the animals were kept in constant

lighting instead of a 12 hour cycle for a period of two months. This may have had an effect on the efficacy of the restraint stress experiments, as the latter are thought to work through a steroid-dependent mechanism. We therefore plan to repeat these experiments, specifically on the *Ribotag/Prestin-CreER^{T2}* and *Ribotag/Sox2-CreER^{T2}* mice.

ii. Determining the appropriate noise exposure for TTS in the *Ribotag/Prestin-CreER^{T2}* and *Ribotag/Sox2-CreER^{T2}* and its efficacy as a pre-conditioning treatment. Mice were exposed to 91, 94, 97, 100 and 105 dB SPL octave-band noise for 2 hours. In addition, one group was exposed to 89 dB SPL for 15 min, 24 hours prior to the noise exposure. Our data show that while 105 dB SPL noise exposure in both strains results in a PTS, 91-97 dB SPL noise exposures result in TTS (Fig. 6). Given the more stable threshold shift at 24h following a 94 dB noise exposure as compared with a 91 dB noise exposure, 94 dB SPL was chosen as the noise exposure to evaluate TTS. To our surprise, the 'canonical' 89 dB pre-conditioning treatment did not result in an improvement in the threshold shift at 8 days (Fig. 6). While this may be strain-specific, we tend to believe that it is possible that the effect, similar to pre-conditioning with stress, is limited to lower noise exposures. We therefore next tested Dexamethasone as a pre-conditioning treatment.

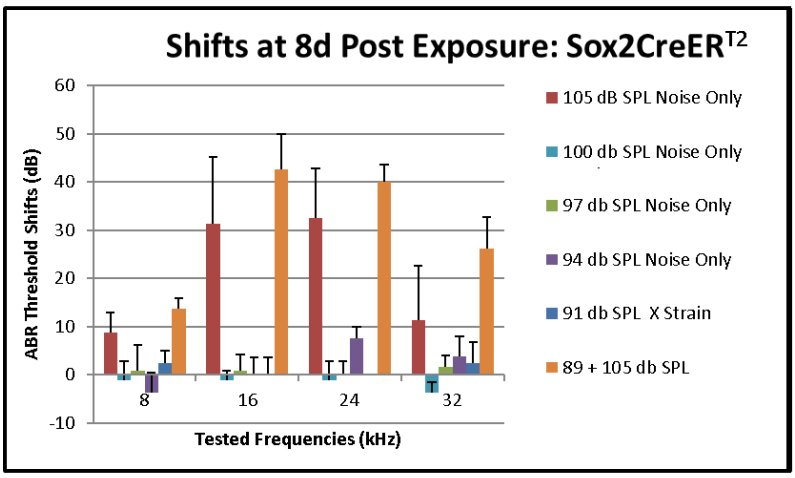


Figure 6 – Threshold shifts 8 days post exposure with and without sound pre-conditioning.

iii. Determining the effect of Dexamethasone pre-treatment on the PTS induced by octave band noise exposure of 102.5 and 105 dB SPL for two hours using the B6CBAF1/J mice. To determine whether Dexamethasone is a likely good pre-conditioning treatment we tested four groups of four mice each, injected with

0.5 mg/kg Dexamethasone IP, or saline, immediately before noise exposure. While the pre-treatment resulted in a slight decrease on the PTS following a 102.5 dB SPL noise exposure, it had no protective effect following a 105 dB SPL noise exposure (data not shown). We decided to continue and test other pre-conditioning treatments prior to settling on the Dexamethasone/Noise exposure/Restraint stress as these treatment efficacies appear to be highly limited depending on the level of noise exposure. SAHA was chosen as a potential better pre-treatment and an amendment was submitted and is currently in review. In addition, we plan to submit an amendment suggesting a direct cross-comparison of pre-treatments for 102.5 and 105 dB noise exposures, as this will be a significant contribution to the field and set clear standards comparing the different pre-treatments in one setting.

b. Subtask 2-4: planned for year II according to the original statement of work.

III. Specific Aim 3: Planned for years II and III according to the original statement of work.

Opportunities for training and professional development provided by the project:

Training –

1. the PI, Ronna Hertzano, participated in a RNA-seq course performed at the NIH. This lead to the identification of new analysis platforms and specifically, familiarity with the Cytoscape environment now used in the laboratory.
2. Zachary Margulies, a technician hired for the project is mentored by the Co-I Didier Depireux. Zachary learned over the first year of the project how to perform and analyze ABR and DPOAE. He is now proficient and performs these tasks routinely.
3. Yoko Ogawa, PhD, a developmental biologist originally trained in the field of zebrafish, who was hired for the project, was trained by Beatrice Milon, PhD, to perform inner ear dissections, cytocochleograms and immunohistochemistry.

Professional Development – the group formed a RNA-seq consultation team which includes Yang Song, an informatics specialist in the Institute for Genome Sciences (IGS), who now attends all laboratory meetings. Through routine weekly meetings, the group was challenged and solved difficulties regarding sequencing of very small amounts of starting material precipitated from RiboTag mice and continues to actively learn and review all literature in the field.

Dissemination of results to communities of interest –

We are working on two publications which we hope to submit in year two of the project. The first is a technical publication focused on RiboTag-seq. The second is a comparative analysis of pre-conditioning treatments in mice to ameliorate the effects of noise exposure.

Plans for the next reporting period –

Our main goals for the next reporting period will be:

1. A complete description of the OHC and SC translatome of adult mice. This will be accomplished by completing the RNA-seq of the processed tissue, data analysis and initial validation.
2. A complete description of the changes in gene expression in OHC and SC as a result of PTS and TTS inducing noise exposures. We will particularly focus in our analyses on the differences in the molecular changes to identify pathways that should be counter-acted using small molecules or medications, for oto-protection.
3. An unbiased comparison of different pre-conditioning treatments for noise exposure, identifying at least one treatment that is significantly more efficacious to focus on in year III of the project.

IMPACT

At this point there is “nothing to report” as we are still waiting for RNA-seq results and final analyses.

CHANGES/PROBLEMS

Change in approach and reason for change

- Pre-conditioning treatments are being compared and will likely be changed. Preliminary data as reported above indicate that some of the canonical pre-conditioning treatments may have limited efficacy with even a mild increase in noise exposure. As we aim to focus on the molecular mechanism of robust pre-conditioning interventions, we will first complete a critical comparison of our initially selected as well as newly reported treatments (e.g. SAHA) prior to pursuing with the molecular analysis.

Actual or anticipated problems or delays and plan to resolve them

- An initial delay occurred due to late ACURO approval. As animal experiments started later than originally planned, we hired the post doc for the project only in January of 2015 and plan to continue working on the project 3-9 months following its official completion.

- As a result of the variability in the efficacy of pre-conditioning treatments, we added a calibration and cross-comparison step. We are not concerned by the delay that this may cause as the quality and significance of the results outweighs this delay.

Changes that have a significant impact on expenditure

- New research published this year indicates an important role for additional cell types following noise exposure. To maximize the use of the tissue obtained from our noise exposed animals, we decided to sequence both the entire cochlea as well as the immuno-precipitated cell type-enriched RNA. This results in a minor increase in the cost of sequencing that will likely be offset by molecularly investigating only the top 1-2 most efficacious pre-conditioning treatments instead of all three as stated in the original plan.

Significant changes in biohazards

- Amendments were submitted to include Dexamethasone and SAHA as pre-conditioning treatments. The Dexamethasone amendment was approved while the SAHA is under review.

Significant changes in care/use of human subjects, vertebrate animals

Nothing to report

PRODUCTS

Publications, conference papers, and presentations

- Our research group published a manuscript in Nature Communications describing the importance of the RFX family of transcription factors in hair cell survival. This work was funded by two RO1 grants from the NIH, DC013817 and DC003544

  Elkon R, Milon B, Morrison L, Shah M, Vijayakumar S, Racherla M, Leitch CC, Silipino L, Hadi S, Weiss-Gayet M, Barras E, Schmid CD, Ait-Lounis A, Barnes A, Song Y, Eisenman DJ, Eliyahu E, Frolenkov GI, Strome SE, Durand B, Zaghloul NA, Jones SM, Reith W, Hertzano R. RFX transcription factors are essential for hearing in mice. Nat Commun. 2015 Oct 15;6:8549

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who work on the project

Name	Ronna Hertzano
Project Role	PI
Researcher identifier	
Nearest person month worked:	2
Contribution to the project	Overall responsibility for the proposal and all aspects of the research program including: hiring and training personnel, ensuring quality of data, interpretation of data, oversight of methods, administrative responsibility and reporting to the DoD.
Funding support	NIH R01, DC013817; Action on Hearing Loss. G65_Bowl; NIH R01, DC003544

Name	Didier Depireux
Project Role	Co-I
Researcher identifier	
Nearest person month worked:	1.2
Contribution to the project	Set up the noise exposure system, ordered the noise exposure box, trained Zachary Margulies, oversees the noise exposure protocols, ABR and DPOAE setup and measurements.
Funding support	MII, Translational Research in Hearing Foundation, Capita foundation, NIH/NIDCR

Name	Ran Elkon
Project Role	Co-I
Researcher identifier	
Nearest person month worked:	1.2
Contribution to the project	Data analysis and study design
Funding support	

Name	Beatrice Milon
Project Role	Research Specialist and supervisor
Researcher identifier	
Nearest person month worked:	6 months
Contribution to the project	Study design, tissue collection, schedule oversight, training of Yoko Ogawa, RiboTag IP and RNA analysis
Funding support	NIH R01, DC013817

Name	Yoko Ogawa
Project Role	Post Doctoral Fellow
Researcher identifier	
Nearest person month worked:	9 months
Contribution to the project	Tissue collection, cytocochleograms, validation (currently setting up), animal care
Funding support	

Name	Zachary Margulies
Project Role	Research Assistant
Researcher identifier	
Nearest person month worked:	12 months
Contribution to the project	Performs all noise exposures, hearing measurements, pre-conditioning, assists with writing animal protocols
Funding support	

Changes in the other support of the PI/ other key personnel:

- The PI and Beatrice Milon, PhD are now supported also by a NIH grant. This does not conflict with the current project. The PI changed effort from 20% to 15%.
- Ran Elkon (Co-I) changed position from a research fellow in the Netherlands Cancer Institute to a Principal Investigator at the Sackler School of Medicine, Tel Aviv University.

Other organizations:

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

- **Quad Chart – see appendix 1.**

APPENDICES

- **Quad Chart**
- **Detailed methods**

Towards a molecular understanding of noise induced hearing loss

Log number: MR130240

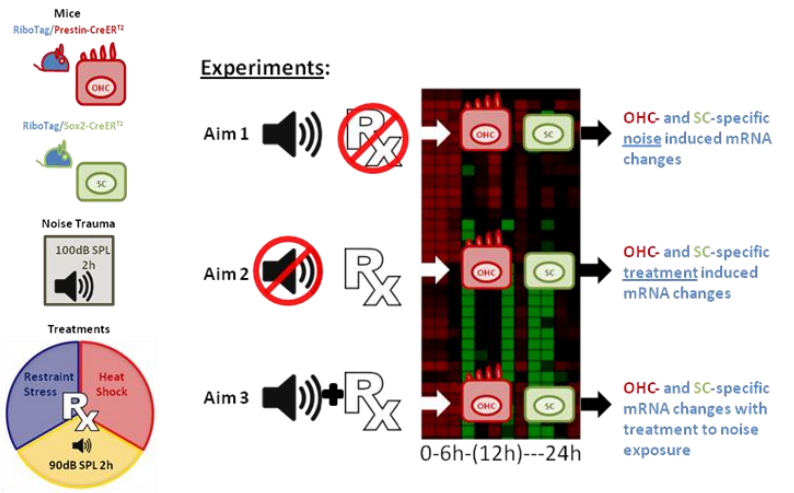
PI: Ronna Hertzano **Org:** University of Maryland School of Medicine **Award Amount:** $1,500,000

Study/Product Aim(s)

- Identification of the principal cells affected by noise trauma – hair cells verses supporting cells.
- Identification, characterization and validation of the cell type-specific translational changes secondary to noise induced hearing loss.
- Identification of the cell type-specific signaling cascades activated during successful treatment of noise induced hearing loss in mice.
- Define at least two key signaling cascades that could be targeted to develop new therapeutic interventions to prevent and treat noise induced hearing loss.

Approach

We established animal models that allow cell type-specific RNA extraction from adult mouse inner ears. We will apply these mice to determine the cell type-specific translational changes at different time points following noise exposure with and without treatment, compared with the baseline translatomes prior to exposure. Identified key regulatory pathways will be validated using real time RT-PCR.



Timeline and Cost

Activities	CY	14-15	15-16	16-17
To establish the OHC- and SC-specific translatome of adult mouse inner ears				
To determine the OHC- and SC-specific transcriptional and signaling cascades activated in response to PTS-inducing noise injury				
To determine the OHC- and SC-specific signaling cascades activated in vivo in response to otoprotective interventions				
To evaluate the OHC- and SC-specific signaling cascades affected *in vivo* in mice that underwent prophylactic otoprotection and subsequently exposed to PTS-inducing noise.				
Estimated Budget ($K)	**$500**	**$500**	**$500**	**$000**

Updated: (Dec 3, 2015)

Goals/Milestones

CY14-15 Goal – OHC- and SC- translatomes at baseline

☐Identification and characterization of the OHC and SC-specific tanslatomes

CY15-16 Goal – Defining the inner ear translatome in response to noise trauma

☐Identification of the key signaling cascades initiated by noise trauma

CY16-17 Goal – In vivo dissection of cell type-specific changes in response to treatment and pre-treated noise exposure

☐Identification and characterization of the signaling pathways induced by otoprotective interventions from noise induced hearing loss

☐Comparison of the cell type-specific molecular changes in response to PTS- and TTS-inducing noise exposure

☐Identification of at least two new pathways for generating new interventions to prevent and treat noise induced hearing loss

Budget Expenditure to Date

Projected Expenditure: 500,000

Actual Expenditure: 331,469.49

DETAILED METHODS

Induction of Cre expression

Progeny from the crosses Prestin$^{CreERT2/CreERT2}$;RiboTag$^{HA/HA}$ x CBA/CaJ and Sox2$^{creERT2/+}$; RiboTag$^{HA/HA}$ x CBA/CaJ are injected with Tamoxifen (Sigma-Aldrich) between P4 and P10. Pups are intraperitoneally (IP) injected with 3 mg of Tamoxifen per 40 g of body weight using a 30G1/2 ultrafine needle insulin syringe (Becton Dickinson) containing a 10mg/ml Tamoxifen solution (Tamoxifen powder is diluted in USP grade corn oil).

Immunoprecipitation (IP) and RNA extraction from RiboTag mice

Dissections

Mice are euthanized one at a time between 9 and 11 weeks of age using CO_2 asphyxiation followed by cervical dislocation. Inner ears are harvested and transferred in cold PBS to extract the cochlear duct. The tissue is snap-frozen in dry ice and store at -80°C until processing.

Solution Composition

Homogenization buffer: 50 mM Tris-HCl pH 7.5, 100mM KCl, 12mM $MgCl_2$, 1% NP-40, 1 mM DTT, 1X Protease Inhibitor Cocktail, 200 U/ml RNAse Inhibitor, 100 μg/ml Cyclohexamide and 100 mg/ml Heparin.

High Salt Buffer: 50 mM Tris-HCl pH 7.5, 300mM KCl, 12mM $MgCl_2$, 1% NP-40, 1 mM DTT and 100 μg/ml Cyclohexamide.

Immunoprecipitation of Polysomes

Cochlear ducts extracted from 4 males and 4 females are pooled and processed for Immunoprecipitation and RNA extraction as described in Sanz et al., 2009. 1 ml of homogenization buffer is used to homogenize the samples in a 2 ml Dounce tissue grinder first with the "loose" pestle, then with the "tight" pestle. The homogenate is centrifuged at 10,000 g for 10 min at 4°C. 50 μl of supernatant is collected and stored at -80^{0}C to serve as the input sample. 5 μg of purified monoclonal anti-HA antibody (Clone HA.11, Enzo Life Sciences) are added to the rest of the supernatant and incubated at 4°C for 6 h with rotation. Following this incubation, the equivalent of 300 μl of Dynabeads® Protein G (ThermoFisher Scientific) are added to the homogenate-antibody sample and incubated at 4°C

overnight with rotation. The next day, using a magnetic rack, the supernatant is discarded and the beads are washed with 800 μl of high salt buffer 3 times for 10 min at 4°C with rotation.

RNA Extraction

We use the RNeasy Plus Micro Kit (QIAGEN) for RNA extraction. Following the last wash with High Salt Buffer, 350 μl of RLT lysis buffer supplemented with β-mercaptoethanol is added to the homogenate-antibody-beads sample and the input and vortexed for 30 sec. The supernatant from the homogenate-antibody sample is collected using the magnetic rack. The input sample is centrifuged at 13000 g for 3 min at room temperature. The IP and input samples are then processed with the RNeasy Plus Micro Kit following the manufacturer's instructions.

Evaluation of RNA Quality and Quantity

1 μl of RNA from IP and input samples is used to evaluate the RNA integrity and quantity with a RNA Pico Chip on a 2100 Bioanalyzer (Agilent).

Assessment of enrichment from cell-type specific RNA

Transcript enrichment obtained after IP is tested by a real time RT-PCR assay. 500 pg of IP and input RNA are used as templates for reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific). The resulting cDNA is amplified by real-time PCR using the Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific) and the following primer pairs: Gapdh Fwd-GGAGAAACCTGCCAAGTATGA Rev-TCCTCAGTGTAGCCCAAGA; Slc26a5 Fwd-GAAAGGCCCATCTTCAGTCATC Rev-GCCACTTAGTGATAGGCAGGAAC; Sox2 Fwd-CAGGAGAACCCCAAGATGCACAA Rev-AATCCGGGTGCTCCTTCATGTG; Pou3f4 Fwd-CTGCCTCGAATCCCTACAGC Rev-CTGCAAGTAGTCACTTTGGAGAA. Real-time PCRs are run on a StepOnePlus Real Time PCR System (Applied Biosystems). Data were analyzed with the ΔΔCt method.

RNAseq

Library preparation

A minimum of 2 ng of RNA is used as input for library preparation with the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®. Library construction and sequencing is performed at the Institute for Genome Sciences of the University of Maryland, School of Medicine (IGS).

Sequencing

Libraries will receive 0.11 a lane of sequencing on an Illumina HiSeq 4000 with a paired-end 150 base-pair configuration.

Cytocochleogram

Dissections

Mice are euthanized one at a time between 9 and 11 weeks of age using CO_2 asphyxiation followed by cervical dislocation. Inner ears are harvested and transferred in cold 4% PFA for 16 to 24 h. Following fixation, inner ears are decalcified in 0.5 M EDTA for 3 days. After decalcification, cochlear sensory epithelia are carefully dissected into apex, apex-middle, middle-base and base to preserve the morphology.

Whole-Mount Immunostaining.

Sensory epithelia are permeabilized in PBS-0.5% tween20 for 1 hour at room temperature followed by blocking in PBS-0.5% tween20 with 5% donkey serum at room temperature. Tissues are then incubated with a goat anti-Prestin antibody (1:200, SC22692, Santa Cruz Biotechnology). Detection is performed with an Alexa Fluor® 546 conjugated donkey anti-goat IgG (1:500, Life Technologies) supplemented with Alexa Fluor® 488 conjugated Phalloidin (1:500, Life Technologies) and 300nM of DAPI (Life Technologies) for one hour at room temperature. Samples are then mounted in anti-fade medium (ProLong® Gold antifade reagent, Life Technologies). Images are acquired with a Nikon Eclipse E600 microscope connected to an Infinity 3 camera (Lumen*era*) and the Infinity Analyze software (Lumen*era*).

Outer Hair Cell Count

A montage of the entire cochlea is performed by assembling consecutive images using Photoshop (Adobe, San Jose, CA) or PowerPoint (Microsoft). Once reconstituted, the length of the cochlea is measured using ImageJ and converted into percentage of distance from apex to base. Outer hair cells are counted throughout the entire length of the cochlea in increments of 10%. Missing outer hair cells are assessed based on the absence of prestin immunostaining. Data is represented as percentage of missing hair cells in each segment of 10%, 0% being the apex and 100% being the base.

Detailed Protocol of Auditory Phenotyping:

Anesthesia

For all pharmaceuticals or substances to be administered to the animals in this protocol, only pharmaceutical-grade chemicals are used in accordance with the SOM policy on pharmaceutical-grade compounds in research animals. As an anesthetic we use pharmaceutical-grade Ketamine:Xylazine.

Prior to injecting the mice with an anesthetic, the mouse is restrained by the person handling the mouse, the abdomen is prepped with a 70% alcohol pad and the mouse held at a 30 degrees angle with its head facing back and down and abdomen up. A tuberculin syringe with a 25G or smaller bore needle is used to administer the anesthetic.

The mice are anesthetized using intraperitoneal injection of Ketamine:Xylazine - Ketamine (80-100 mg/Kg) and Xylazine (5-10 mg/Kg) solution. The maximal volume administered to any mouse is 0.3 ml IP. Xylazine in this mixture is at a concentration of 20 mg/ml. Xylazine is available at concentrations of 20 mg/ml and 100 mg/ml. If using 100 mg/ml the volume of Xylazine would be 0.2 ml with a volume of 1.8 ml of WFI (water for injection). The volume of Ketamine would remain the same.

Anesthesia is monitored to ensure an appropriate depth. To monitor depth of anesthesia, a pedal withdrawal reflex is performed by lightly extending the leg and pinching the foot. Only when not withdrawing it is determined that the mouse is under sufficient anesthesia. Lacri-lube is placed on the corneas to prevent a corneal injury. Respiratory rate and depth are visually inspected. To avoid hypothermia during the procedure and during recovery, the mice are kept on a heating pad (Deltaphase Isothermal pad or Moist/Dry heat pad with temperature control.

Auditory Brainstem Response and Distortion Product Otoacoustic Emissions

Hearing thresholds are determined at 8, 16, 24 and 32 kHz using an ABR recording system (Tucker-Davis technologies TDT Bioacoustic System RZ6). All recordings are performed in a soundproof reverberant box. The experiments are performed in the HSF1 Animal Facility, room 643A. Before starting the experiment the mouse is anesthetized as described. Anesthesia record is kept for each animal to record response to pedal withdrawal test and indicate if additional anesthesia is required. Subdermal needle electrodes (27G bore needles) are placed at several points to measure electrical activity transmitted from the inner ear into the brainstem. One recording electrode is attached to each superior postauricular area of each ear. A reference electrode is placed at the vertex (base of skull) between the two ears. A ground electrode is attached to the base of the tail.

The ABR sound stimuli are delivered through a Fostex FT17H speaker, which is secured 10 cm in front of the anesthetized mouse. The ABR speaker sound levels are calibrated every morning before any ABR

recordings are taken in order to ensure consistent and accurate sound levels. Sound stimulus levels are calibrated at the 10 cm distance point from the speaker with a 1/4-inch PCB condenser microphone. 512 sweeps of 2.5 ms long bursts are introduced to the mouse ears at varying intensities beginning at 90 dB SPL (Sound Pressure Level) and proceeding in 5 dB decrements down to 0 dB SPL. ABR stimulus and resulting waveforms are controlled, monitored, and assessed within the BioSigRZ software (TDT). Thresholds are defined by a visual review of waveforms to be the lowest level at which a repeatable peak pattern could be discerned. Mice are tested 2-5 days before noise exposure and 24 hours, 8 days, and 15 days post-exposure. At the termination of measuring the hearing thresholds, Distortion Product Otoacoustic Emissions (DPOAEs) are evoked. DPOAEs are evoked by playing two primary tones simultaneously and the resulting emissions are measured with an Etymotic ER-10B+ device. The DPOAEs are collected 6 times per second at 8 kHz, 12 kHz, and 24 kHz.

Once the test is completed the electrodes are removed from the mouse and the mouse is transferred to a separate cage. Once the mouse fully recovers and is ambulating in the cage (not any sooner), is it returned to its routine housing. Prior to putting the mouse back in its original cage, the injection site and sites of electrode placement are inspected for swelling/bleeding. In the event of abnormal appearance of the site (swelling, bleeding, tenderness, decreased ambulation or appearance of pain and withdrawn activity), the veterinarian is consulted. A similar inspection is performed in the post-operative days (one day after the procedure and routinely three times a week). The injection/electrode sites are inspected one more time during the week after the procedure to minimize animal handling, unless the mouse exhibits behavior suggestive of pain or distress as described above. The amount of anesthetic administered is recorded and these records are saved.

The duration of the procedure is about 60-80 minutes including the administration of anesthesia, ABR, DPOAE, and mouse recovery.

Noise exposure

Mice are placed, un-anaesthetized and unrestrained, into a cage with multiple compartments (one animal per compartment).The cage is 5.2”W x 8.3”L x 5.2”H. The cage is positioned inside of a reverberant noise exposure box. A noise stimulus, described as octave band of noise (8-16 kHz), is presented for 2 hours. The noise stimulus (ranging 89 dB SPL to 105 dB SPL depending on the experiment) is delivered through the Fostex FT17H speaker, which is secured into the ceiling of the noise exposure box. Noise exposure sound levels are measured before every noise exposure with a calibration

microphone in order to ensure consistent and accurate sound levels; sound levels are tested in multiple locations throughout the cage to ensure uniform distribution of noise stimulus. Furthermore, the noise stimulus is generated and presented within the SigGenRZ software (TDT). Following noise exposure mice are immediately returned to home cages. See Figure 7 below for the noise exposure cage positioned within the noise exposure box and beneath the speaker.

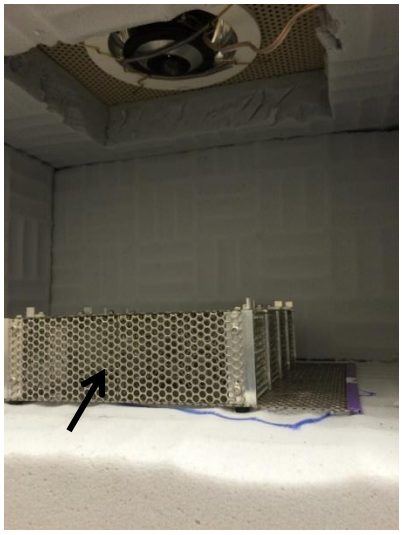


Figure7 – Noise exposure cage (black arrow) placed inside the reverberant noise exposure box.